Reference: *Biol. Bull.*, 139: 549–556. (December, 1970)

COELOMOCYTE AGGREGATION IN *CUCUMARIA FRONDOSA*: EFFECT OF ETHYLENEDIAMINETETRAACETATE, ADENOSINE, AND ADENOSINE NUCLEOTIDES

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Coelomocytes, a collective name for cells existing within the coelomic fluid of echinoderms, rapidly aggregate upon removal from the coelomic cavity. Descriptive accounts of the aggregation phenomena have been given by various workers (Bookout and Greenburgh, 1940; Boolootian and Giese, 1959; Endean, 1966), and a striking feature is the morphological change which occurs in one of the coelomocyte types, the bladder amoebocyte, and which appears to involve extensive membrane rearrangement.

Ethylenediaminetetraacetate (EDTA) was first used as a disaggregating agent by Anderson (1953) and is thought to act by chelating with divalent cations, in particular, calcium and magnesium, which, at the turn of the century, were shown to be of importance in cell aggregation (Roux, 1894).

Recently, much interest has been centered around the effects of adenosine and adenosine nucleotides on the aggregating behavior of cells; for example, adenosine diphosphate (ADP) has been shown to enhance platelet aggregation which can be reversed by adenosine or 2-chloroadenosine (Born and Cross, 1963), and adenosine triphosphate (ATP) has been found to inhibit the aggregation of embryo chick fibroblast cells (Knight, Jones, and Jones, 1966). Adenosine nucleotides have been implicated as playing an important physiological role in cellular adhesive and aggregating mechanisms which appear to reside at the cell surface (Jones, 1966).

In this study an attempt has been made to measure quantitatively the effects of EDTA, adenosine, and adenosine nucleotides upon coelomocyte aggregation in the holothurian, *Cucumaria frondosa*.

Materials and Methods

Sea cucumbers weighing between 238 g and 425 g were obtained from Logy Bay, Newfoundland. They were kept in running sea water of temperature range 1° C to 3° C during the course of these experiments, which lasted from January to April. All needles and glassware coming into contact with coelomic fluid were siliconized with Siliclad (Clay-Adams). The substances to be tested for their effect upon coelomyocyte aggregation were used at the following concentrations: EDTA, 6×10^{-3} *M*; ATP, ADP, and adenosine monophosphate (AMP), all at 5×10^{-4} *M*. They were dissolved in an artificial sea water (ASW), calcium and magnesium free, of the following composition: sodium chloride, 28.326 g;

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sodium sulfate, 3.917 g; potassium chloride, 0.664 g; sodium bicaronate, 0.192 g; and potassium bromide, 0.096 g per liter of double-distilled water. Calcium- and magnesium-free sea water was used because EDTA and, to a lesser degree, ATP and ADP chelate with these divalent cations. If these compounds were dissolved in ordinary sea water, it would be difficult to assess what effective concentration of these compounds was coming into contact with coelomic fluid and coelomocytes.

Particluate matter was removed from the solutions by filtering through a millipore filter of 0.45 μ pore size. The pH of the solutions was adjusted to either pH 6.0 or pH 7.8 using 0.1 *N* HCl and 0.1 *N* NaOH, respectively, and was checked prior to each experiment. The pH of the resulting mixture of coelomic fluid and test solution was not measured.

Coelomcyte samples were obtained by piercing the dorsal body wall with a 22-gauge needle and withdrawing 0.1 ml of coelomic fluid into a disposable tuberculin syringe containing 0.1 ml of one of the test solutions. The two solutions were mixed by moving the plunger in the syringe gently to-and-fro several times. A drop of fluid was then placed in a chamber which was made as follows: two strips of Parafilm (Fisher), 1 cm apart, were placed on a microscope slide, and a glass coverslip was carefully lowered on top of the drop of fluid, with its edges resting upon the strips of Parafilm. The resulting chamber was sealed with Vaseline. At no time was the drop of fluid allowed to come into contact with either the Parafilm strips or Vaseline seal. The strips of Parafilm prevented the weight of the coverslip from causing damage to the cells.

Twenty minutes after the initial withdrawal of the coelomic fluid, the percentage of all cells that were not associated with a cell aggregate, that is, the free cells, was determined in the chamber. Of these free cells, the ratio, expressed as a percentage, of the number of bladder amoebocytes to the sum of bladder and filiform amoebocytes was enumerated in order to assess the tendency of the cells to undergo morphological change. The number of cell aggregates and the number of cells per aggregate were recorded as an indication of the intensity of the cellular aggregation process.

The chambers, solutions, needles, and syringes were kept at 4° C, except when the sample was withdrawn and the counting of the cells was done in the chambers. A minimum of 200 cells, both free and in aggregates, were counted. The same six animals were used for each solution at each pH value.

Results

The effects of EDTA, ADP, and ATP upon coelomocyte aggregation are summarized in Table I. Of all the substances tested, only EDTA at pH 6.0 prevented both the morphological change from the bladder to filiform amoebocyte and the aggregation of coelomocytes. EDTA at pH 7.8 largely prevented cell aggregation but did not prevent the morphological change. Adenosine triphosphate at pH 7.8 appeared to enhance the morphological change of bladder to filiform amoebocyte. The results obtained with the remaining substances were not statistically significant from those obtained with ASW (Ca^{++} and Mg^{++} free) controls. The effect of pH alone upon coelomocyte aggregation was not found to be statistically significant when the data obtained for ASW (Ca^{++} and Mg^{++} free) at pH 6.0 and pH 7.8 were compared ($P > 0.05$).

TABLE I

Effects of EDTA, adenosine and adenosine nucleotides upon coelomocyte aggregation

	pH	Of all cells % free	Of free cells B/B + F × 100†
ASW*	6	65.82 ± 5.81	7.67 ± 2.27
	7.8	39.54 ± 9.72	4.98 ± 1.53
ASW* + EDTA 6×10^{-3} *M*	6	89.70 ± 5.45**	93.87 ± 2.26***
	7.8	79.76 ± 7.39**	10.37 ± 2.04
ASW* + Adenosine 5×10^{-4} *M*	6	53.64 ± 9.50	7.98 ± 2.91
	7.8	58.67 ± 7.82	1.83 ± 1.15
ASW* + AMP 5×10^{-4} *M*	6	54.25 ± 6.16	4.36 ± 3.09
	7.8	52.88 ± 8.03	1.22 ± 0.88
ASW* + ADP 5×10^{-4} *M*	6	46.84 ± 11.24	6.72 ± 3.87
	7.8	51.63 ± 9.04	0.68 ± 0.45
ASW* + ATP 5×10^{-4} *M*	6	56.18 ± 8.06	3.64 ± 2.71
	7.8	47.31 ± 3.50	0.21 ± 0.20**

† Bladder amoebocyte/bladder + filiform amoebocyte × 100.
* ASW—Artificial sea water Ca^{++} Mg^{++} free.
** $0.01 > P > 0.001$ significant
*** $P < 0.001$ highly significant
} With respect to ASW* at appropriate pH.
Mean ± S.E.M. 6 animals.

The percentage of all the cells counted that were found in a given aggregate is shown in Figure 1. The intensity of the cell aggregation process was found to be minimal with EDTA at pH 6.0, and this finding is in keeping with the results described for EDTA at pH 6.0 upon the morphological change and cell aggregation. The very small number of aggregated cells obtained with EDTA at pH 6.0 were bladder amoebocytes, which were probably adjacent to one another purely by chance in the counting chamber and therefore were counted as a cell aggregate.

Coelomocyte morphology

It is relevant to this study to describe the more predominant coelomocyte types and to present the morphological changes which occur prior to and during cell aggregation. Figures 2–11 show these morphological changes as well as the major coelomocyte types.

The bladder amoebocyte (Figs. 2 and 3) is 15 μ–20 μ in diameter and possesses petaloid or bladder-like lobes which appear to undergo extensive morphological rearrangement to what is known as the filiform amoebocyte (Figs. 4 and 5) as a prerequisite to cell aggregation. Morula cells, appoximately 10 μ in diameter, are capable of movement and are characterized by several refractile granules within their cytoplasm when viewed with phase-contrast (Fig. 6). Figures 7 to 9 show motile cells which can exhibit a variety of different forms, and these have been called "lymphocytes" (Hetzel, 1963) because of their morphological and staining

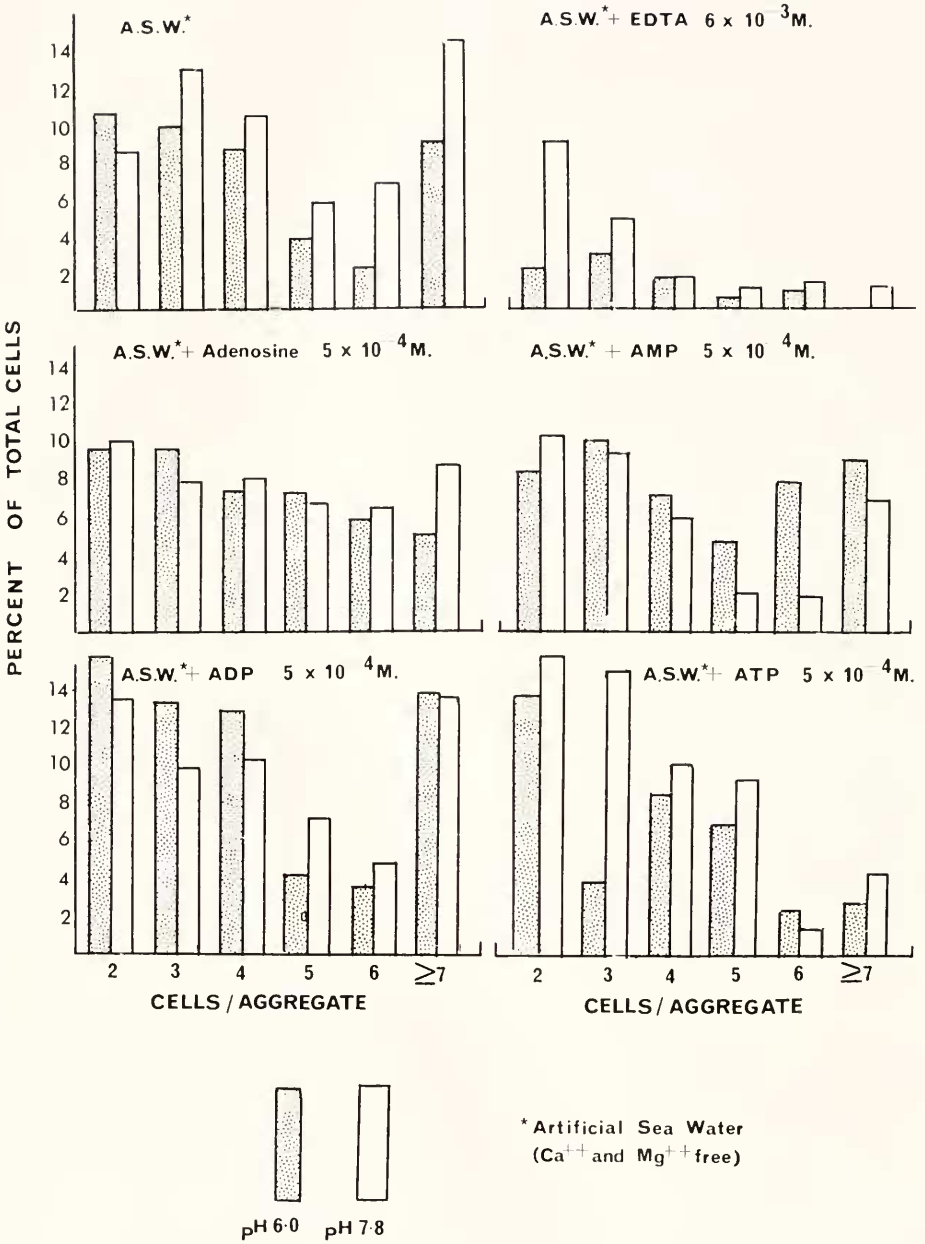

FIGURE 1. The percentage of total cells counted found in a given aggregate.

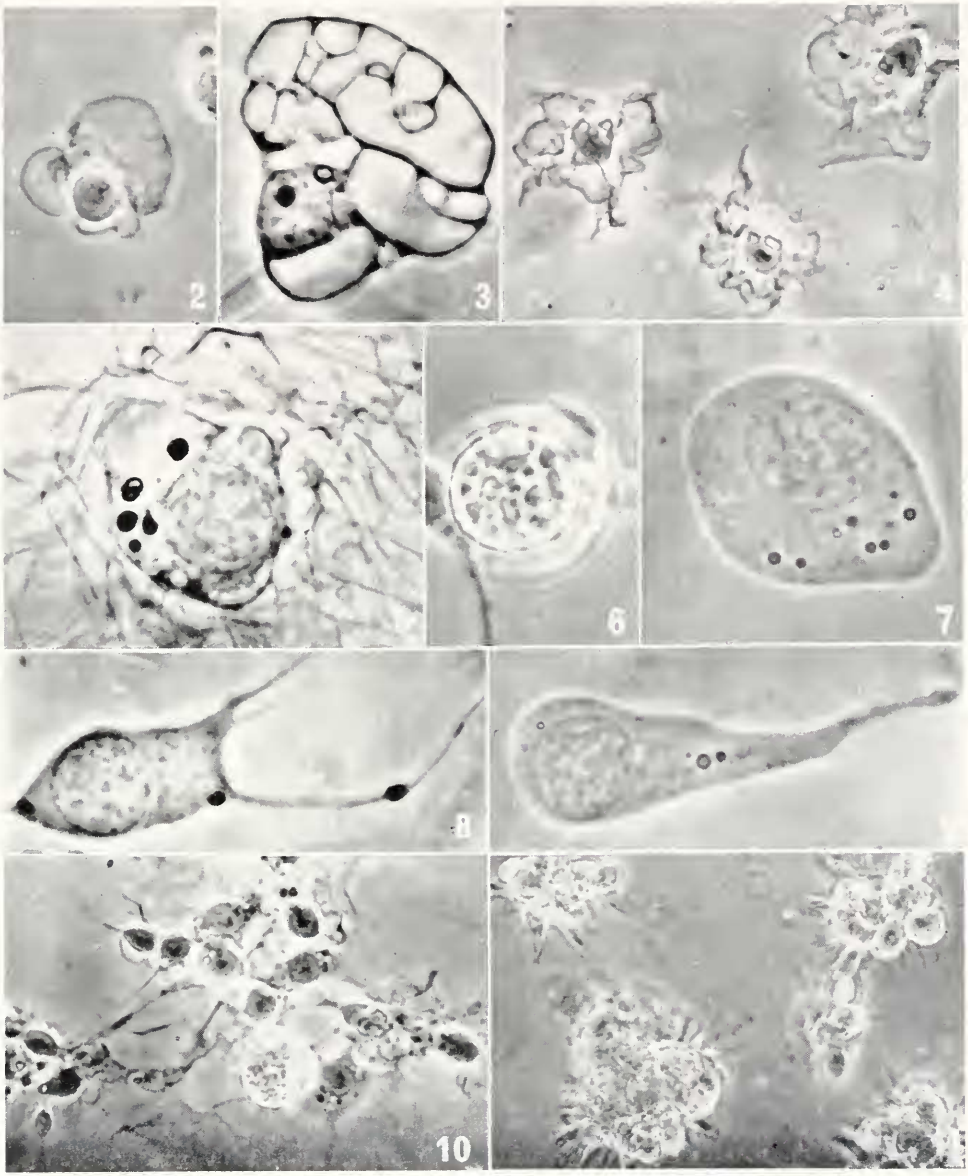

FIGURE 2. All photographs phase-contrast; Bladder amoebocyte, × 400. FIGURE 3. Bladder amoebocyte, × 1000. FIGURE 4. Filiform amoebocyte, × 400. FIGURE 5 Filiform amoebocyte, × 1000. FIGURE 6. Morula cell, × 1000. FIGURES 7, 8 and 9. Lymphocytes, × 1000. FIGURES 10 and 11. Typical coelomocyte aggregation, × 400.

similarities to mammalian lymphocytes. This is an unfortunate choice of terminology since it implies that these cells are analogous to mammalian lymphocytes which are known to be responsible for cell mediated and humoral immune mechanisms, properties which have not yet been shown to be possessed by echinoderm "lymphocytes". It is important to note that lymphocytes are notoriously deficient in morphological details, a fact contributing to the difficulties encountered in higher animals or associating the many physiological properties observed with this morphologically classified type of cell. Until such time that the known functions of echinoderm "lymphocytes" will suggest a functional terminology, a term should be used which denotes our ignorance regarding the functions of these cells and not one which implies properties as yet unproved.

Typical coelomocyte aggregates are shown in Figures 10 and 11.

Discussion

It has been shown that EDTA at pH 6.0 prevents both the morphological change and cell aggregation. At this pH, EDTA is reported to have a low stability coefficient for binding calcium ions, the greatest binding capacity for calcium being at pH 8.0 (Curtis, 1967). In this study, EDTA at pH 7.8 prevented aggregation but did not prevent the morphological change from bladder to filiform amoebocyte. These differences suggest that the cell aggregation phenomena can be divided into two different stages; one possibly requires calcium ions for the actual aggregation process, while the morphological change, and consequently cell aggregation, is prevented either by the removal of some other material which is chelated by EDTA at pH 6.0 or by EDTA reacting directly with the cell membrane or some other cellular component. In this regard it is pertinent to recall that L. Weiss (1960) pointed out that effective disaggregation of cells by EDTA is not proof that it does so by chelating with calcium ions.

Jones (1966) has recently put forward a unifying hypothesis of cell adhesion linking the adenosine nucleotides with contractile and relaxing properties of an actomyosin-like protein with ATPase activity located at the cell surface. For example, levels of ATP and ADP, by governing the physiological state of the contractile protein could, by initiating conformational changes in the membrane, bring about changes in the distribution of charges at the cell surface and hence alter adhesiveness and aggregating ability of cells. Evidence has been obtained using a variety of different cellular systems to support this concept. For example, glycerol-extracted cells of non-muscular origin, contracted in the presence of exogenous ATP (Hoffman-Berling, 1954), and contractile proteins have been isolated from many different cell types (Hoffman-Berling, 1956; Bettex-Galland, and Luscher, 1959; Loewy, 1952); ATPase activity has also been demonstrated in membranes of cells (Essner, Novikoff, and Masek, 1958; Novikoff, 1960).

No effect of adenosine or adenosine nucleotides upon coelomocyte aggregation was found in this study, with the possible exception of ATP at pH 7.8, which appeared to enhance the morphological change. One explanation of this latter result is that ATP provided an additional source of energy necessary for the membrane rearrangement which occurs in the bladder amoebocyte. An attempt was made to use a potentially more sensitive method of observing the effects of adenosine and adenosine nucleotides upon coelomocyte aggregation, that is, the

turbidimetric method of Born and Cross (1963) which has been used extensively to follow aggregation of mammalian cells. However, in order to standardize the number of coelomocytes per cubic millimeter of fluid it was necessary to prevent aggregation by using EDTA at pH 6.0. Subsequent addition of adenosine and adenosine nucleotides did not produce aggregation as measured by a decrease in optical density and by direct microscopical observation. This finding is, of course, in keeping with the data presented in this study. The interpretation of this result is complicated, however, by the observation of Born and Cross (1963), who showed that platelets would not aggregate in response to ADP if the cells were in plasma which contained EDTA as an anticoagulant. One could argue that the presence of EDTA prevented any subtle effects of adenosine and adenosine nucleotides upon aggregation from being detected.

That adenosine and adenosine nucleotides, when used at concentrations which are effective in modifying avian and mammalian cell aggregation, fail to modify coelomocyte aggregation raises the question as to whether or not the mechanism of coelomocyte aggregation is similar to that postulated for other cell aggregating systems (Jones, 1966). To answer fully this question, studies are needed to see if contractile and relaxing proteins having ATPase activity can be isolated from coelomocyte membranes.

The result obtained with EDTA at pH 6.0 is also contrary to the known effects of EDTA at this pH upon mammalian and other cell aggregating systems. Many reports on the failure of EDTA to prevent cell aggregation and the enchancement of cell dispersion have been attributed to its poor binding capacity for calcium ions at this pH (Curtis, 1967). The mechanism by which EDTA at pH 6.0 stabilizes bladder amoebocyte membranes warrants further investigation.

The author is indebted to Dr. K. B. Roberts, Professor of Physiology, for his advice and encouragement and critical reading of the manuscript. My thanks also go to Dr. F. A. Aldrich, Director of the Marine Sciences Research Laboratory, who so generously granted facilities for this work.

Summary

Cells found within the coelomic cavity of the sea cucumber, *Cucumaria frondosa*, aggregated rapidly upon removal from the body. This cell aggregation is normally characterized by a morphological change in the bladder amoebocyte which becomes a filiform cell. A method has been devised whereby the effects of EDTA, adenosine, and adenosine nucleotides could be observed upon coelomocyte aggregation. It was found that EDTA at pH 6.0 in ASW (Ca^{++} and Mg^{++} free) prevented the morphological change from bladder to filiform amoebocyte as well as cell aggregation. EDTA at pH 7.8 in ASW (Ca^{++} and Mg^{++} free) did not prevent the morphological change although it did prevent cell aggregation. Based on the different chelating affinities of EDTA for calcium ions at these two different pH values, it is suggested that coelomocyte aggregation has two components: (1) the morphological change, which is Ca^{++} independent and possibly requires the removal of some other cation or is a direct effect of EDTA upon some

cellular component, and (2) the cell aggregation itself which appears to be Ca^{++} dependent.

Adenosine and adenosine nucleotides in general did not appear to influence coelomcyte aggregation. These results raise the question as to whether or not the mechanism of coelomocyte aggregation is similar to and can be described in the same terms as the mechanism currently used to explain avian and mammalian cell aggregation.

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